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Distribution of Animal Drugs between Skim Milk and Milk Fat Fractions in Spiked Whole Milk: Understanding the Potential Impact on Commercial Milk Products

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Supporting Information

ABSTRACT: Seven animal drugs [penicillin G (PENG), sulfadimethoxine (SDMX), oxytetracycline (OTET), erythromycin (ERY), ketoprofen (KETO), thiabendazole (THIA), and ivermectin (IVR)] were used to evaluate the drug distribution between milk fat and skim milk fractions of cow milk. More than 90% of the radioactivity was distributed into the skim milk fraction for ERY, KETO, OTET, PENG, and SDMX, approximately 80% for THIA, and 13% for IVR. The distribution of drug between milk fat and skim milk fractions was significantly correlated to the drug's lipophilicity (partition coefficient, log *P*, or distribution coefficient, log *D*, which includes ionization). Data were fit with linear mixed effects models; the best fit was obtained within this data set with log *D* versus observed drug distribution ratios. These candidate empirical models serve for assisting to predict the distribution and concentration of these drugs in a variety of milk and milk products.

KEYWORDS: *veterinary drug residues, cream, antibiotics, anthelmintics, NSAID, partitioning, distribution, milk*

INTRODUCTION

The U.S. Food and Drug Administration (FDA) recently developed a multicriteria-based ranking model for risk management of animal drug (henceforth termed drug) residues in milk and milk products.[1] The model was completed as part of the overall effort to improve an already strong and effective regulatory system for milk and milk products. The model was developed to serve as a decision-support tool to assist with re-evaluating which drug residues should be considered for inclusion in milk testing programs.[1] While as part of the FDA's science-based approach to food safety, the model considered a wide range of data and information, including government-conducted surveys, the published literature (e.g., data on the impact of temperature and processing techniques), elicited expert comment, and information from an external peer review, but actual data on drug residue distribution in milk products were scarce.

It has long been recognized that contaminant concentrations in foods may be impacted by food processing steps that change the fat composition of the food product. For example, Mann et al.[2] examined DDT concentrations in milk products derived from raw (nonprocessed) milk known to be contaminated with DDT and showed that the concentration of DDT in each milk product was essentially determined by the percentage of milk fat in the product. Indeed, it is universally recognized that when highly lipophilic environmental contaminants are found in foods, they are concentrated in the lipid compartment of that food.

Many drugs are not highly lipophilic, and there are limited experimental data about the distribution of drug residues during processing of milk products. Among published studies, the distribution of residues of a single drug into one or two milk products was examined, typically into cheeses.[3−11] A more expansive product study was conducted by Cerkvenik et al.,[12] examining the distribution of ivermectin (IVR) residues in ewe's milk and milk products that included yogurt, fresh cheese, ripened cheese, whey, and albumin cheese. The authors reported the relationships between IVR concentrations in the product and percentages of both milk fat and solids.[12] These experiments did not include measurements of the distribution of ivermectin in any fat-containing products absent of protein, which makes it difficult to determine if the drug was associated with lipid or protein. To the best of our knowledge, only one paper to date examined the distribution of as many as four antibiotics in milk, and this study was post-intramammary infusion or intramuscular injection of the radiolabeled compound in goats.[13]

The research described here tested the hypothesis that if whole milk contains a drug its subsequent distribution among the fractions of milk could be predicted on the basis of drug lipophilicity. The studies were conducted using whole milk

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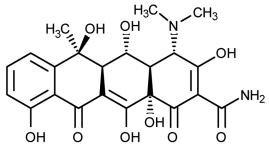
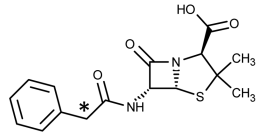
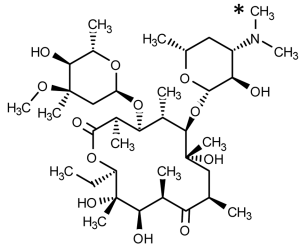
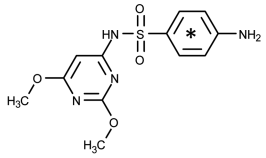
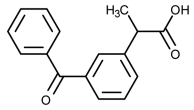
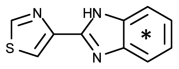
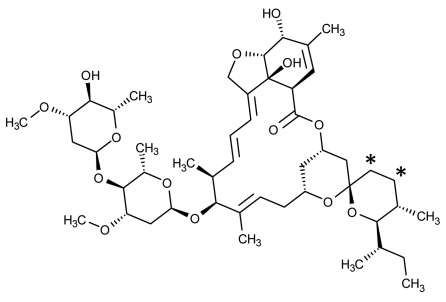
Table 1. Drug Structures and Physicochemical Properties

Compound	Class/Use	M.W. S.A. (nCi/nmol)[a]	log P[b]	pKa[b]	log D[b]
 ^{3}H(G)-Oxytetracycline (OTET)[c]	Tetracycline/ Antibiotic	460.1 g/mol 1,000 (20nM) 100 (200nM) 10 (2,000nM)	-1.50	7.75	-2.50 (6.8) -4.25 (7.4)
 ^{14}C-Penicillin G (PENG)[d]	β-Lactam/ Antibiotic	372.5 g/mol 55 (20/ 200nM) 5.5 (2,000nM)	1.67	3.53	-1.60 (6.8) -1.70 (7.4)
 ^{14}C-Erythromycin A (ERY)[d]	Macrolide/ Antibiotic	733.9 g/mol 55 (20/ 200nM) 5.5 (2,000nM)	2.83	8.38	1.24 (6.8) 1.69 (7.4)
 ^{14}C-Sulfadimethoxine (SDMX)[e]	Sulfonamide/ Antibiotic	310.3 g/mol 28 (20 /200nM) 2.8 (2,000nM)	1.48	6.91	1.23 (6.8) -0.49 (7.4)
 ^{3}H(G)-Ketoprofen (KETO)[c]	NSAID/ Analgesic	254.3 g/mol 1,050 (20nM) 105 (200nM) 10.5 (2,000nM)	2.81	3.88	-0.11 (6.8) 0.06 (7.4)
 ^{14}C-Thiabendazole (THIA)[d]	Fungicide/ Anthelmintic	201.3 g/mol 19.9 (75/ 200nM) 1.99 (2,000nM)	2.93	12.47	2.93 (6.8) 2.47 (7.4)
 ^{3}H-Ivermectin B1a (IVR)[d]	Avermectin/ Anthelmintic	875.1 g/mol 500 (20nM) 50 (200nM) 5 (2,000nM)	6.61	12.4	6.61 (6.8) 6.21 (7.4)

[a]Specific activities (S.A.) were adjusted depending on dose and isotope, as indicated. Values in parentheses are nominal concentrations. [b]Log *D* (pH 7.4) and log *P* accessed from www.chemspider.com on January 28, 2015, using the ACD Lab-predicted values. Values for log *D* at pH 6.8 were calculated using log *P* from ChemSpider (see above) and pK_a values from the Drug Bank (www.drugbank.ca/) accessed February 11, 2015. [c]Radioactively labeled with a single, general tritium atom. [d]Compound radioactively labeled with a directed label, specified on the structure with an asterisk. An asterisk within an aromatic ring indicates a uniform label on the ring.

spiked with drugs instead of *in vivo* incorporation to have more consistent starting drug concentrations to facilitate modeling efforts. Ziv and Rasmussen[13] reported a similar distribution of antibiotics in skim milk, whether administered intravenously or

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by intramammary infusion. The research described herein was undertaken to determine the distribution of drug residues in milk and milk products to improve our understanding of the potential impact of residues in commercial milk products. Specifically, the experiments studied the distribution of seven drugs into skim milk and milk fat fractions derived from pasteurized whole cow milk. It was also the intent of this research to develop an empirical (based on experimental data) model that can be used to determine the distribution of other drugs or drug metabolites into milk and milk products. Drugs examined in this study span four classes of antibiotics and also include two anthelmintics and one analgesic. Data from six of the seven drugs provide the first measurements of this kind. The experiments focus on characterizing the distribution of drug residues in postpasteurized, but otherwise unprocessed, cow milk among milk fat and skim milk fractions. A separate study underway will provide additional information about the distribution of drug residue in curd, whey, and associated proteins.

MATERIALS AND METHODS

Selection of Drugs and Concentrations in Pasteurized Whole Milk. The criteria for choosing drugs to be studied were as follows: (1) drugs being evaluated in the FDA 2015 ranking model (or closely related analogues),[1] (2) drugs that spanned a wide range of lipophilicity (log P = 1.5−6.6) to examine its effect on distribution behavior, and (3) the availability of radiolabeled compounds so that the distribution of drugs and their degradates could be easily tracked in different fractions. We selected penicillin G (PENG), a β-lactam antibiotic; sulfadimethoxine (SDMX), a sulfonamide antibiotic; oxytetracycline (OTET), a tetracycline antibiotic; erythromycin (ERY), a macrolide antibiotic; ketoprofen (KETO), a nonsteroidal anti-inflammatory drug (NSAID); thiabendazole (THIA), a fungicide and anthelmintic; and ivermectin (IVR), an anthelmintic (Table 1). Drug structures, the site of the radiolabel, the specific activity (S.A.), and physicochemical properties are also listed in Table 1.

The lowest drug concentration (20 nM) in pasteurized whole milk examined for these experiments was selected to be both relevant (e.g., in the range of typical regulatory values when available) and practical (e.g., large enough that the distribution between the two phases could be quantified). The other two concentrations were formulated to be 10-fold (200 nM) and 100-fold (2000 nM) higher, allowing for evaluation of the concentration dependence of drug distribution. Unlabeled drug was added to the radiolabeled chemical to produce sufficient chemical mass (^{14}C) or to lower specific activity (^{3}H) of final dose solutions (Table 1).

Safety. Radiolabeled chemicals were handled in compliance with Nuclear Regulatory Commission (NRC) regulations for ^{14}C and ^{3}H.

Chemicals, Supplies, and Equipment. Raw cow milk was obtained from the bulk milk tank at the North Dakota State University Dairy Unit farm (≤48 h in storage postmilking). Reference standards used to validate compositional analyses of various milk fractions were obtained from Eurofins DQCI (Mounds View, MN) and were used prior to the expiration date. Samples of unlabeled drugs were obtained from Sigma-Aldrich (St. Louis, MO) and radiolabeled drugs as follows: SDMX from Sigma-Aldrich; PENG, OTET epimer, ERY, IVR, and KETO from American Radiolabeled Chemicals, Inc. (ARC) (St. Louis, MO); and THIA from Moravek Biochemicals (Brea, CA). It was discovered that the tritiation reaction with unlabeled OTET resulted in a product consisting of a 3:1 ratio of unlabeled OTET epimer to [^{3}H]OTET epimer as determined by liquid chromatography−mass spectrometry (LC−MS). Proton nuclear magnetic resonance was used to confirm the ARC starting material was OTET. The precursor ion and fragment ions of the [^{3}H]OTET product were +2 of a separate unlabeled OTET standard, as determined by LC−MS/MS, although retention times were different. Differing retention times indicate epimerization/isomerization. The epimer could not be identified, though 4-epi-OTET was ruled out, as retention times differed. However, doses were diluted with unlabeled OTET (Sigma) at ratios of 1:5 (20 nM), 1:50 (200 nM), and 1:500 (2000 nM). Silica gel plates for thin layer chromatography (TLC) were purchased from Analtech (Newark, DE) and polypropylene tubes (50 mL) from Sarstedt, Inc. (Newton, NC). The following equipment was used: a Precision water bath (Thermo Scientific, Milford, MA) for pasteurization, a MagniWhirl water bath (BlueM Electric Co., Blue Island, IL) for drug equilibration, and an Allegra X-14R centrifuge (Beckman-Coulter, Brea, CA) for separation of the milk fat fraction from the skim milk fraction. Radioactivity was quantified using a Tri-Carb 1900 TR liquid scintillation counter (LSC, Packard, Meridan, CT) for liquid samples, a model 307 sample oxidizer (Packard) for solid sample combustion generating $^{14}CO_2$ or $^{3}H_2O$, and a AR-2000 Imaging Scanner (Bioscan, Washington, DC) for purity analysis by TLC. Scintillation cocktails include Ecolite (MP Biochemicals, LLC, Solon, OH) for liquid samples, Carbosorb and Permafluor (both from PerkinElmer, Waltham, MA) for oxidized solid samples to trap $^{14}CO_2$, and Monophase to trap $^{3}H_2O$ (PerkinElmer).

Milk Processing and Radiochemical Analysis. On the morning of each set of experiments, 2 qt (1.89 L) of raw milk were obtained and 12 tubes (50 mL each) were prepared. These tubes, as well as two tubes of nanopure water (20 mL each), were then pasteurized in a 63 °C water bath for 30 min, while being shaken at 100 rpm. After pasteurization, triplicate whole milk tubes were spiked with 100 μL of stock solutions of radiolabeled drug (10 000, 100 000, and 1 000 000 nM) or solvent [solvent concentrations never exceed 0.2% (Table S1a)], to yield a final concentration of 0, 20, 200, or 2000 nM (except for THIA, 75 nM was substituted for the 20 nM dose because of its low S.A.). Whole milk in the context of this study is defined as cow milk that has been pasteurized but otherwise unprocessed. Drug was added to two nanopure water tubes at a final concentration of 200 nM to evaluate any effect of the milk matrix on the putative decomposition of the drug. After each tube had been vortexed, a 1 mL aliquot was removed for LSC analysis (100 μL in triplicate) to determine the starting radioactivity for radiochemical mass balance calculations. Each drug was equilibrated in the milk or water mixture by being shaken in a 38 °C water bath (to mimic body temperature) for 30 min. The 30 min equilibration time was determined to be sufficient to establish a steady state distribution in the milk fractions in a separate set of experiments (Figure S1), which examined the drug distribution at 0.5, 1, 2, and 4 h. Following the equilibration period, a 300 μL aliquot was removed from the 2000 nM tube to establish drug integrity postequilibration by TLC analysis, and then all tubes were centrifuged (4000g for 45 min at 35 °C) to separate milk fat from pasteurized whole milk. The milk fat layer was removed from the top of skim milk by spatula, yielding on average 2.2 g of milk fat fraction, with a mean of 46.4 mL of skim milk fraction remaining. Aliquots of skim milk (3 × 200 μL) were assayed by LSC for quantification based on total radioactivity, and by TLC to qualitatively investigate possible degradation of drug during processing. To obtain homogeneous milk fat fractions that did and did not contain drug, samples required heating in a water bath at 38 °C for 30 min, and vortexing for 3 min until they were homogenized to butter consistency, prior to combustion and LSC analysis (5 × 0.1 g aliquots).

For TLC analysis, proteins in skim milk aliquots (1 mL) were denatured with acetone (3 mL), vortexed, centrifuged at 380g, and decanted. The acetone layer was evaporated with nitrogen, the residue was reconstituted with 50 μL of methanol, applied onto a 5 cm × 20 cm silica gel plate, and chromatographically separated with a mobile phase specific for each drug (Table S1). Some compounds, such as OTET and THIA, were not directly amenable to TLC without additional cleanup of the extract and required solid phase extraction (SPE, method in SI) before TLC analysis. Aliquots from the milk fat fraction (0.5 g) were treated the same as aliquots of the skim milk fraction, except acetone extracts were produced by sonication for 1 h at room temperature. Semiqualitative assessments of skim milk and milk fat fraction extracts for metabolites and degradates were performed by TLC and radioactivity monitoring (RAM).

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Table 2. Mean Compositional Analysis of Whole Milk, Skim Milk Fraction, and Milk Fat Fraction of All Drug Distribution Studies and Literature Goat Milk Values from a Related Study in the Literature[a]

	lipid %[a]	total solid %[a]	total N %	true protein %[b]	casein protein %[c]
whole milk[d]	3.75 ± 0.19	12.37 ± 0.15	3.18 ± 0.09	3.00 ± 0.10	2.48 ± 0.08
min	3.36 (4.51)	12.15 (14.22)	3.02	2.83	2.36
max	4.05 (9.78)	12.68 (20.16)	3.33	3.17	2.62
skim milk[d]	0.25 ± 0.05	9.03 ± 0.17	3.16 ± 0.13	2.98 ± 0.14	2.42 ± 0.11
min	0.17 (0.11)	8.71 (10.0)	2.89	2.71	2.18
max	0.35 (0.17)	9.37 (11.3)	3.36	3.19	2.58
fat	81.58 ± 2.95	83.78 ± 2.29	1.09 ± 0.07	n/a	n/a
min	76.44 (77.8)	80.14 (85.8)	0.93	n/a	n/a
max	86.36 (83.1)	87.96 (92.4)	1.23	n/a	n/a

[a]Values in parentheses are for goat whole milk, skim milk, and cream from ref 13. [b]True protein % calculated on the basis of total non-protein N %. [c]Casein protein % calculated on the basis of total non-casein N %. [d]Means and ranges are reported for compositional analysis. For whole milk, $n = 7$ different milk samples obtained from the North Dakota State University dairy on different dates (June 2014 to January 2015) used in seven different drug distribution studies, analyzed in triplicate. For skim milk and milk fat, means are analyses of three separate preparations of skim and cream samples from each of the seven whole milk samples.

Milk Compositional Analysis. Compositional analyses were performed on blank (0 nM) samples of (a) pasteurized whole milk, (b) skim milk fractions, and (c) milk fat fractions to provide lipid, total solid, and protein percentages, which allowed for both monitoring of raw milk variability and week to week reproducibility of sample preparations. Aliquots from the 0 nM tubes were removed for total solids (1 mL of whole milk or skim milk fraction, 0.1 g of milk fat fraction), lipid composition (5 mL of whole milk or skim milk fraction, 0.25 g of milk fat fraction diluted with 5 mL of water), and protein determination (25 mL of whole milk or skim milk fraction, 0.5−1.0 g of milk fat fraction). Total solid determinations of whole milk, skim milk, and milk fat fractions were made gravimetrically by drying to a constant weight. The lipid content of whole milk, skim milk, and milk fat fractions was determined by adapting the AOAC modified Mojonnier lipid extraction method (half the sample and solvent amounts, AOAC Method 989.05).

Protein determinations on 0 nM milk blanks were performed according to accepted AOAC methods for total nitrogen (Method 991.20), non-protein nitrogen (Method 991.21), and non-casein nitrogen (Method 998.05) using Tecator heating block digestion (Foss, Eden Prairie, MN). Exceptions to the AOAC methods included using two Pro-Pac TT-35 tablets (Alfie Packers, Inc., Omaha, NE) containing preweighed instead of individually weighed K_2SO_4, $CuSO_4$, and TiO2; 15 not 20 mL of sulfuric acid; an addition of a 30 min room temperature incubation; heating for 60 min at 420 °C with no temperature ramp; addition of 90 not 85 mL of water to digestion tubes after heating; and Kjeldahl titrations performed on a Kjeltec 2300 instrument (Foss) using a 0.1142 N H_2SO_4 titrant performed at North Dakota State University's Nutrition Laboratory.

Statistical Analyses. Standard statistical methods and measures were used in the analyses of data, including estimations of means, variability, and significance of observed differences and/or trends. The potential dose dependence of the observed drug distribution ratio $[Drug]_{milk\ fat}/[Drug]_{skim\ milk}$ was evaluated with linear regression analyses where data was significant if $p \leq 0.05$. In the case where the observed drug distribution was mainly in the skim (OTET, PENG, ERY, SDMX, KETO, and THIA), drug residue in milk fat was corrected for the remaining skim milk volume and associated drug concentration, and drug residue in skim milk was corrected for the remaining lipid percent. In a similar manner, where the observed drug distribution was mainly in the milk fat (IVR), drug residue in skim milk was corrected for the remaining milk fat volume and associated drug concentration, and drug residue in milk fat was corrected for the remaining skim milk volume. Relationships between the log distribution ratios (observed and corrected ratios) and lipophilicity (log P and log D, which includes ionization where relevant) were analyzed with linear mixed effects models (REML, R Development Core Team, Vienna, Austria), where concentration and phase were fixed effects, and replicate within concentration was a random effect. Replication consisted of three independent equilibrations per concentration. Models were compared with the quality of fit measure Akaike information criterion (AIC), where smaller AIC values signify better fit models.

RESULTS AND DISCUSSION

Compositional Analyses. Processing of an average of 48.6 ± 0.90 mL of whole milk yielded an average of 46.4 ± 0.16 mL of skim milk fraction and 2.2 ± 0.05 g of milk fat fraction (7 different whole milk samples, 12 replicates each, over a 7 month period). The compositional analyses (Table 2) for the milk samples and fractions (skim milk and milk fat) were consistent across time despite seasonal and silage changes for cattle. With the exception of lipid analysis in skim milk, where the coefficient of variance value (COVs) was 20%, all other COVs from analyses were ≤6%. Compositional analyses of NDSU milk compared closely to other Midwest U.S. milk analyses (Table S1) and ranged between 94 and 103% of the values from various regional dairy herds analyzed by Eurofins. The outliers for the range were non-protein and non-casein values, which reflect concentrations of <1%. Values for percent protein (3.18) and fat (3.75) for whole milk were also in agreement with the national standardized lactation averages for Holstein in 2013 (3.06 and 3.73% for protein and fat, respectively).[14] As expected, milk fat fraction preparations were closer to commercially prepared butter (defined in Section 321a of the Federal Food, Drug, and Cosmetic Act as "containing not less than 80 per centum by weight of milk fat")[15] than commercially prepared heavy cream (≥36% milk fat, 21 CFR § 131.150)[16] and dry cream (≥40%, but <75% by weight of milk fat on an as is basis, 21 CFR § 131.149).[16] Our milk fat preparations were best characterized as a semisolid state. Milk fat fraction preparations from goat milk prepared in a manner similar to that described in this study reported similar lipid percentages for these fractions, ranging from 77.8 to 83.1% (Table 2).[13]

Raw whole and skim milk samples with known composition (i.e., fat, total solids, and protein percentages) were purchased from Eurofins and analyzed in our lab to verify QA/QC conditions of our weekly analyses (Table S2). All study values differed from Eurofins values by <4% for lipid, total solid, true

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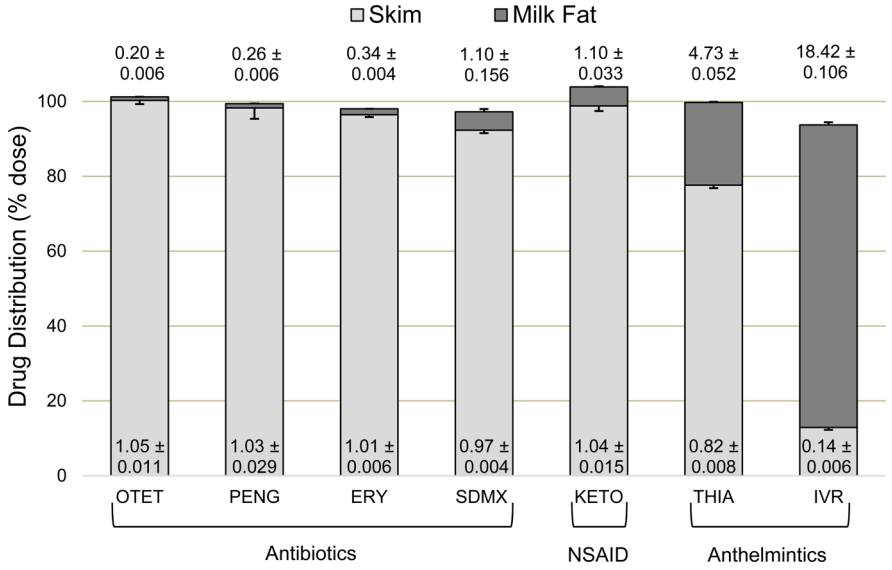

Figure 1. Drug distribution and relative concentration ratios from whole milk into skim milk and milk fat fractions. Bars represent the percent mean of all concentrations (n = 3 concentrations, 3 replicates per concentration) ± the standard deviation of the three dose means based on disintegrations per minute (dpm) of skim milk and milk fat fractions compared to whole milk dpm. Values on the graph represent the mean ratio of the drug concentration in the fraction (milk fat or skim milk) to the initial drug concentration in whole milk ± SD of means between doses (n = 3 mean dose ratios). The sum of stack plot represents total drug recovery.

protein, and casein protein analyses (Table S2), with the exception of the lipid analysis of skim milk, which was present at extremely low concentrations (<0.3% of total).

Drug Stability. The study approach quantified radioactivity in skim milk and milk fat to determine the drug partitioning, assuming the radiolabeled drug remained intact. Therefore, it was imperative to verify the integrity of radiolabeled drug in processed final fractions. TLC chromatograms of all extracts demonstrated that the seven drugs were all stable under the conditions selected (data not shown). Where published data were available that evaluated the effect of temperature on stability, our data were in agreement. No degradation was observed for PENG in milk heated at 40 °C for 10 min.[17] Some inactivation (9%) was observed at higher temperatures (60 °C for 30 min),[17] which suggests minimal degradation would have occurred during our 30 min equilibration at 38 °C. In another stability study of the effects of milk pasteurization (62 °C and 30 min), a 23.6% reduction in OTET and an 8.2% reduction in PENG residues were observed.[18] However, Hassani et al.[19] observed that OTET was thermally stable in milk until 121 °C (<1% reduction) but quickly degraded at >135 °C for 15 s (>56%). Sulfonamides may be even more stable. Heat treatment of sulfamethazine at 65 °C for 30, 45, or 60 min resulted in no significant change in concentration.[20] Sulfadimidine residues did not change under nonindustrial pasteurization conditions (65 °C and 30 min) but were reduced by approximately 5% when stored at 27 °C for 24 h in milk.[21] Using ewe's milk obtained following subcutaneous administration, IVR experienced no loss during normal milk pasteurization[12] and was even stable during high-temperature pasteurization (80 °C for 1 min) and under boiling conditions (10 s at 100 °C).[22]

Adetunji[11] examined changes in PENG, streptomycin, and tetracycline residue levels during yogurt production in multiple Pakistani processing facilities. Slight decreases in concentrations were observed in yogurt processing when going from powdered milk to finished yogurt.[11] However, there were no clear specifications for processing conditions and sampling protocols to surmise whether the same sampling stream was analyzed or what pasteurization conditions were used. Grunwald and Petz[23] observed decreases of PENG residues during yogurt production, and heating (90 °C for 15 min) was identified as a factor that contributed to this decline.

Drug Distribution. The percentage of dose that distributed into the milk fat fraction for the seven drugs tested ranged from <1% of the dose (e.g., OTET, very hydrophilic) to ∼80% of the dose (e.g., IVR, very lipophilic) among the seven drugs examined (Tables S3−S9 and Figure 1). Drug concentrations in milk fat when compared to those in whole milk were 0.2−18 times higher (Tables S3−S9 and Figure 1), indicating some drugs can concentrate into high-lipid content products. Data were highly reproducible, with COV values for the replicates well under 10% for all drugs at all concentrations examined. There was excellent agreement between initial equilibration time study results and distribution study results. Results for both the skim milk fraction and the total dose recovery from equilibration studies (20 nM dose) were within 3% (±1.4% SD) of the subsequent mean drug distribution studies (mean of all doses, calculations not shown) for all drugs. Results between experiments were slightly more variable for the milk fat fraction, within 8% (±5.8% SD) of the all dose mean. Because these two sets of experiments (equilibration and main distribution studies) for each drug were typically performed a few weeks apart, the agreement of these data demonstrated high reproducibility of experimental data. Drug recoveries throughout the distribution experiments, using the 30 min equilibration time, were high for all drugs studied [93−105% of the spiked dose (Figure 1 and Tables S3−S9)]. The dependency of the dose on drug distribution was evaluated over the wide range of doses examined. Linear regression analysis found, at most, a small change in the estimated distribution ratio for PENG and KETO, representing a <1% change across the 100-fold concentration range (Table S10). No other drug displayed any dependence on concentration.

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Table 3. Comparison of Skim Milk and Milk Fat Fraction Partitioning Postmammary Infusion of Antibiotics in Goats[a] to *in vitro* Partitioning in Cow Milk[b]

	PENG				TET[c]	OTET	TET	OTET	SPIR[d]	ERY	SPIR	ERY
	ratio (fraction/whole)[a]	current study ratio[b]	% of dose[a]	current study %[b]	ratio (fraction/whole)[a]	current study ratio[b]	% of dose[a]	current study %[b]	ratio (fraction/whole)[b]	current study ratio[b]	% of dose[a]	current study %[b]
milk fat												
low dose	0.51	0.26	4	1	0.66	0.20	5	1	0.94	0.34	7	2
high dose	0.32		2		0.42		3		0.35		3	
skim milk												
low dose	0.74	1.03	68	100	0.68	1.05	62	101	0.88	1.02	81	97
high dose	0.81		74		0.94		86		0.92		84	

[a]Data from ref 13, dose concentration ranges for PENG, TET, and SPIR are 718 to 44910 nM, 4950 to 276076 nM, and 5338 to 46026 nM, respectively. [b]Current study nominal concentrations were 20, 200, and 2000 nM, with the exception of OTET low dose was 75nM. No dose effect was found in current study, therefore only one set of values is reported for ratio and % of dose. [c]Tetracycline (TET), similar in structure to OTET. [d]Spiramycin (SPIR), similar in structure to ERY.

Table 4. Linear Mixed Effects Model Parameters and Quality of Fit Values Based on Drug Bank pK_a's

dependent variable	independent variable	slope	slope standard error	intercept	intercept standard error	AIC
$\log([Drug]_{milk\ fat}/[Drug]_{skim\ milk})$[a]	log *P*	0.35	0.10	−0.68	0.32	−210.6
$\log([Drug]_{milk\ fat}/[Drug]_{skim\ milk})$[a]	log *D*	0.31	0.05	−0.18	0.15	−214.6
$\log([Drug]_{lipid}/[Drug]_{aqueous})$[b]	log *P*	0.65	0.11	−1.77	0.36	−150.0
$\log([Drug]_{lipid}/[Drug]_{aqueous})$[b]	log *D*	0.51	0.09	−0.78	0.27	−149.1

[a]Observed distribution ratios. [b]Corrected distribution ratios.

Available literature about the residue distribution for these drugs in milk and milk products is scant. In agreement with our findings on SDMX, Rasmussen[24] observed that sulfonamides were distributed mostly to the aqueous milk fraction, called the fat-free phase of milk.[13] Also, our IVR results coincided with what Cerkvenik et al.[12] observed, where IVR concentrated in milk products containing the milk fat. For example, Cerkvenik et al.[12] showed a strong correlation (r^2 = 0.98) of increasing IVR concentration to increasing milk fat content in various milk-derived products, such as bulk milk, yogurts, cheeses, and whey. However, Cerkvenik et al.[12] could not distinguish whether drug residue was associated with milk fat or solid content.

Ziv and Rasmussen[13] infused goat mammary glands with antibiotics (PENG, tetracycline, and spiramycin), collected whole milk, and partitioned it into skim milk and cream by methods similar to that described here. The infused doses (based on body weight) of PENG, tetracycline, and spiramycin along with the $[Drug]_{skim\ milk}/[Drug]_{whole\ milk}$ or $[Drug]_{milk\ fat}/[Drug]_{whole\ milk}$ concentration ratios were compared to the data from this study in Table 3. The tetracycline distribution would be expected to be comparable to the OTET distribution, because their physicochemical properties are similar. Similarly, ERY chemically resembles spiramycin, and both belong to the same antibiotic class. When considering their data (concentration ratios or the % of dose), the high-dose data are most similar to those of the study presented here.[13] Variability in results could be due to the larger range and higher lipid content reported for the goat's milk (4.5−9.8%) versus the 3.8% found in the study presented here (Table 2). The time between intramammary infusion of spiramycin and milk collection was not reported; however, this period would involve complexities of absorption of the drug into the mammary tissue and release back into the milk of fat globules surrounded by a plasma membrane.[25] In contrast, our approach utilized straightforward *in vitro* experiments. The majority of radiolabeled PENG, spiramycin, and tetracycline was found in the skim milk with intramammary infusion,[13] which is in agreement with the findings for PENG, ERY, and OTET in the study presented here.

Chlortetracycline administered by intramammary infusion to goats was found to concentrate in the skim milk fraction from the first milkings,[26] consistent with our findings for OTET [>99% of dose in skim milk (Figure 1)]. Similar to OTET and PENG findings in the current experiments, Hammainen et al.[27] investigated sodium or procaine salts of PENG, OTET, chlorotetracycline, and tetracycline by intramammary infusion and direct addition to milk. Their data demonstrated higher drug concentrations in the aqueous phase compared to the levels in other milk products, i.e., cream or butter. A slow release form of PENG, benzathine PENG, yielded concentrations in skim milk approximately twice those in cream.[28]

Development of Empirical Models for Predicting Drug Distribution. The lipophilicity of a drug can be characterized by the log *D* or log *P*, where log *D* differs in value from log *P* when the drug has ionizable functional groups (e.g., weak acid or base) and the pH of the solution is such that a significant fraction of the drug ionizes. Values for log *D* were available from the ChemSpider Database[29] for pH 5.5 or 7.4, but because the pH of the experimental milk was pH 6.8, log *D* was also calculated using the Drug Bank[30] pK_a and ChemSpider[29] log *P* values as reported in Table 1. For the purpose of comparison, the log *D* values at pH 7.4 are also included in the table. The equations for these calculations were[31]

$$\log D_{acid} = \log P + \log[1/(1 + 10^{pH-pK_a})] \quad (1)$$

$$\log D_{base} = \log P + \log[1/(1 + 10^{pK_a-pH})] \quad (2)$$

The accuracy of the calculated log *D* values depended on the correctness of the pK_a values used. Some drugs had multiple pK_a values, and there was variability in reported pK_a's for some

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J. Agric. Food Chem. 2016, 64, 326−335

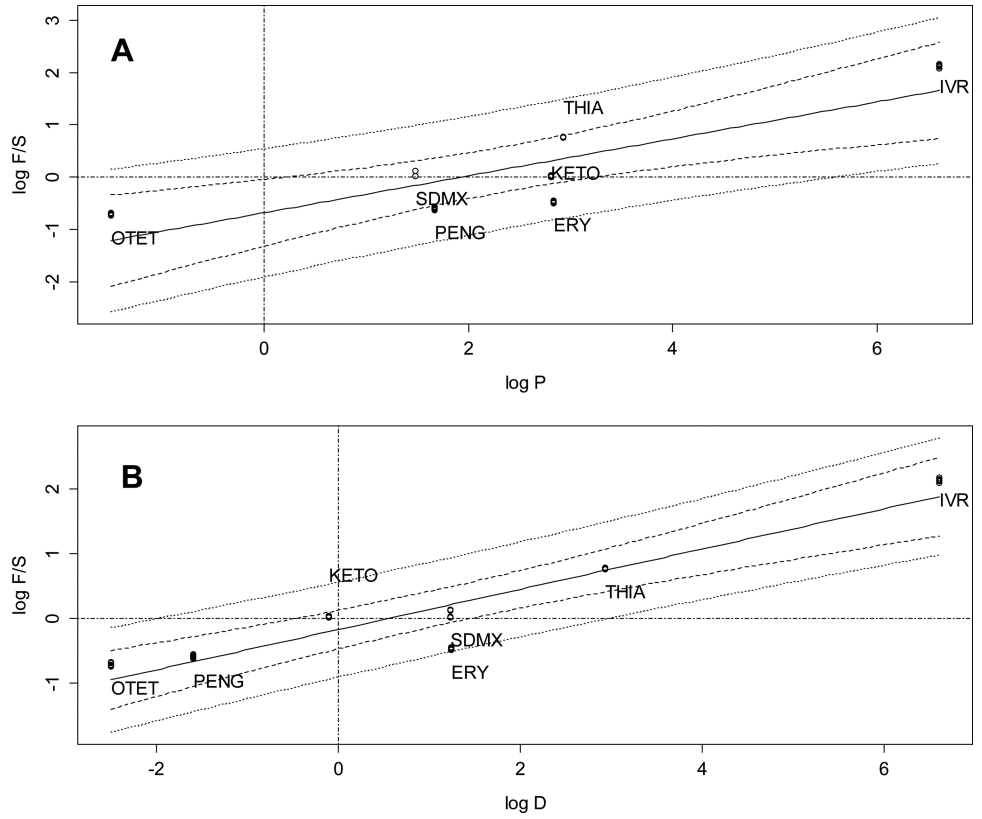

Figure 2. Regression analyses of observed log([Drug]$_{milk\ fat}$/[Drug]$_{skim\ milk}$) (log F/S) vs log P and log D (pH 6.8). Plot A is the regression analysis of log F/S vs log P with a natural y intercept. Plot B is the regression of log F/S vs log D (pH 6.8) with a natural y intercept. Drug Bank pK_a's accessed on February 11, 2015 (www.drugbank.ca). Log P accessed from Chemspider on January 28, 2015 (www.chemspider.com). Calculated using log D_{acid} = log P + log[1/(1 + 10^{pH-pK_a})] or log D_{base} = log P + log[1/(1 + 10^{pK_a-pH})].

drugs at the same pH. For instance, pK_a values for erythromycin ranged from 8.1 to 9.1.[32−37] Hence, for consistency, the pK_a values were all taken from Drug Bank.[30]

The relationship between the log of the observed distribution ratios for all seven drugs and lipophilicity, as described by log P or log D, was evaluated with a linear mixed effects model. All data were used in the model, nine distribution ratios per drug obtained from three replicates at each of three concentrations for each drug. This model was chosen over a simple linear regression model because it takes into account the two stages of sampling (three concentrations and three replications). Model parameter estimates and quality of fit measures are listed in Table 4. Graphs illustrating the fits, including 95% confidence intervals, are shown in panels A (log P) and B (log D) of Figure 2. Also shown in the graphs are the 95% prediction intervals for these models. The empirical model (Figure 2) derived from observed distribution ratios and log D values provided a better fit to the data [small AIC (Table 4)] than the model derived using log P values, reflecting the fact that many of the drugs examined ionize to some extent in milk (pH 6.8).

To assess the contribution of incomplete fraction separation (milk fat containing skim milk and skim milk containing lipid), distribution ratios were corrected for cross-contamination of both fractions (Tables S3−S9). These concentration corrections revealed that within the experimental precision of the study, OTET radioactive residues could not be distinguished between residual skim milk and lipid within the milk fat fraction. Therefore, OTET corrected lipid concentrations were calculated at half the limit of radiochemical detection (LOD = 0.048 μCi/kg). The residual concentrations of all other drugs in each phase could be calculated; therefore, the observed concentrations were corrected without LOD assumptions. These data were also fit with linear mixed effects models described in Table 4 and illustrated in panels A and B of Figure 3. On the basis of the AIC values [log P = −150.0, and log D = −149.1 (Table 4)], the models with the corrected distribution ratios were not different from each other because of the increased uncertainty introduced by the correction. Within this data set, the overall best fit was obtained from log D (calculated from Drug Bank[30] pK_a and ChemSpider[29] log P values) using the observed distribution ratios (Figure 2B and Table 4).

Slopes of the observed (log P vs log D) and corrected (log P vs log D) models differed by 11−21%, and all slopes were significantly lower than 1, indicating that a larger fraction of these drugs distributed to the skim milk fraction than expected on the basis of octanol:water coefficients. Distribution data were not the result of the presence of degradates, as none were detected based on TLC of each of the milk fat and skim milk extracts. The lower slopes (<1) were probably due to the differences between octanol:water and whole milk matrices, which include proteins, sugars, ions, and micelles. Additional experiments currently underway are probing the extent of protein binding among these drugs in the skim milk fraction and may better explain drug distribution within the complex milk matrix. An additional factor could be the higher temperature of these experiments (38 °C) versus the standard temperature (25 °C) used for partitioning (octanol:water) calculations. Studies of partition coefficient temperature dependence suggest that over this small temperature range, values differ by <10%.[38]

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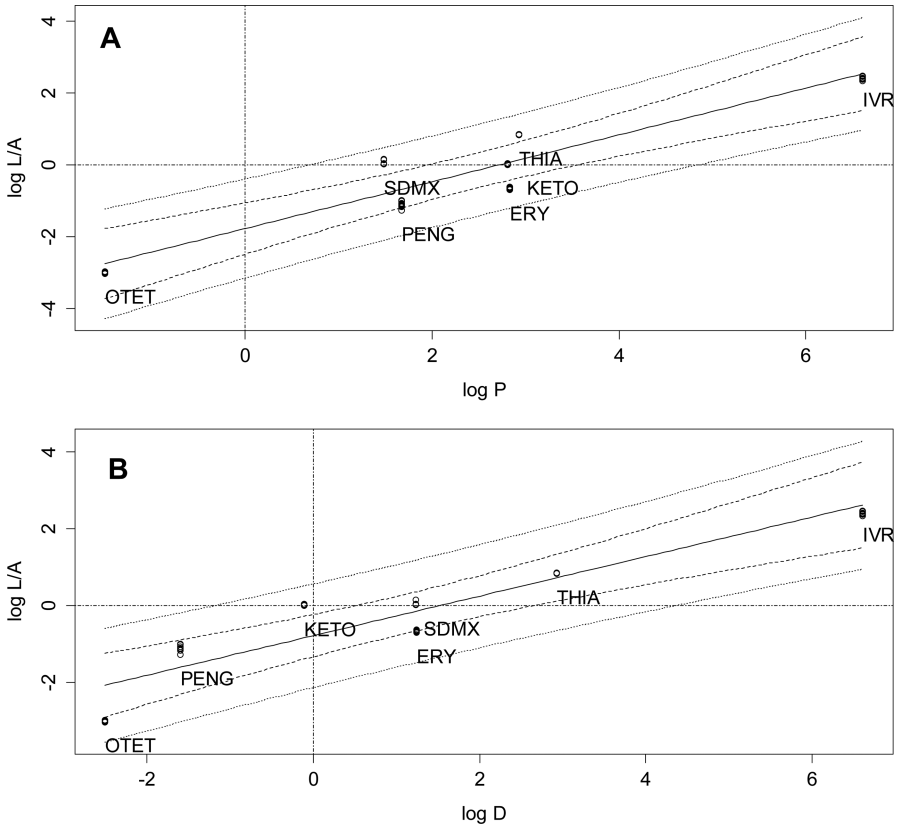

Figure 3. Regression analyses of corrected log([Drug]$_{lipid}$/[Drug]$_{aqueous}$) (log L/A) vs log P and log D (pH 6.8). Plot A is the regression analysis of log L/A vs log P with a natural y intercept. Plot B is the regression of log L/A vs log D (pH 6.8) with a natural y intercept. Drug Bank pK_a's accessed on February 11, 2015 (www.drugbank.ca). Log P accessed from Chemspider on January 28, 2015 (www.chemspider.com). Calculated using log D_{acid} = log P + log[1/(1 + 10^{pH-pK_a})] or log D_{base} = log P + log[1/(1 + 10^{pK_a-pH})].

In summary, these data describe the distribution of seven animal drugs in milk fat and skim milk fractions of cow milk, the first report for six of these drugs. The observed ratio of drug concentration in the milk fat fraction relative to skim milk fraction ranged from 0.10 to 136 (corrected ratio range of 0.05 to 232, excluding OTET). These data indicate that OTET, PENG, or ERY, if present in milk and not eliminated or transformed by processing, will be mostly distributed in nonfat milk products, whereas THIA or IVR residues are distributed in mostly milk fat and as such will concentrate into high-lipid content milk products. On the basis of these data, SDMX and KETO concentrated equally between skim milk and milk fat. The distribution of these drugs in other milk products may be driven by other forces. Models describing the relationships between log D and the log of the observed distribution ratio between milk fat and skim milk fractions, and the corrected distribution ratios for lipid and aqueous phases have been determined. These models serve not only to predict the distribution properties of other animal drugs (or metabolites if the log P or log D is known) in skim milk and milk fat fractions, but should also assist in the prediction of drug residue concentrations in other milk products. More data are needed to better understand the relationship established in these experiments describing the distribution of drug residues in the complex milk matrix.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b04726.

A paragraph on SPE cleanup for OTET and THIA samples and a figure of drug distribution time course equilibration and tables of TLC conditions, Eurofins compositional information, time course equilibration and dose statistics, and the complete set of experimental data for these experiments organized by drug (PDF)

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Notes

The authors declare no competing financial interest.

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J. Agric. Food Chem. 2016, 64, 326−335

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List of Supporting Information

Paragraph on SPE clean-up of OTET and THIA samples.

Table S1a. Solvent conditions for compound storage and incubations.

Table S1b. TLC conditions for each drug (standard or in milk matrix) tested.

Table S2. QA/QC laboratory compositional analysis of Eurofins DQCI whole and skim milk samples.

Table S3. OTET mean, standard deviation, and relative standard deviation values for drug data for three initial concentrations in whole milk.

Table S4. PENG mean, standard deviation, and relative standard deviation values for drug data for three initial concentrations in whole milk.

Table S5. ERY mean, standard deviation, and relative standard deviation values for drug data for three initial concentrations in whole milk.

Table S6. SDMX mean, standard deviation, and relative standard deviation values for drug data for three initial concentrations in whole milk.

Table S7. KETO mean, standard deviation, and relative standard deviation values for drug data for three initial concentrations in whole milk.

Table S8. THIA mean, standard deviation, and relative standard deviation values for drug data for three initial concentrations in whole milk.

Table S9. IVR mean, standard deviation, and relative standard deviation values for drug data for three initial concentrations in whole milk.

Table S10. Linear regression analysis of log $[Drug]_{milk\ fat}/[Drug]_{skim\ milk}$ for different dose levels (20, 200, 2000 nM).

Table S11. Values used for regression analysis of log [Drug]milk fat/[Drug]skim milk vs log P and log D (6.8) of all data used for graphs and calculations from multiple databases.

Figure S1. Drug distribution from whole milk into skim milk and milk fat fractions at four different equilibration times.

Solid Phase Extraction (SPE) Clean-up for OTET and THIA in Skim Milk for TLC

One mL of skim milk was extracted with 4 mL of acetone, vortexed, and proteins precipitated by centrifugation. Acetone extract was taken to dryness under N_2, 37°C, and resuspended in 4 mL of freshly prepared buffer (0.038 M citric acid monohydrate, 0.105 M sodium phosphate dibasic, 0.009 M ethylenediamine tetraacetic acid (EDTA), and 0.18% formic acid; pH 6.8). OASIS HLB (Waters, Milford, MA) SPE cartridges were conditioned with 3 mL each of MeOH, nanopure H2O, and buffer. Sample was loaded onto the column using a 50 mL syringe barrel, 1 ml at a time. Column was washed with 50 mL buffer, followed by 1 mL of 5% MeOH. Drug was eluted in two 1mL aliquots of 100% MeOH, volume reduced under nitrogen, and then loaded on TLC plates. Chelation of minerals with OTET prevented discrete migration (smears on plate) requiring TLC plates to be pretreated with 10% EDTA

Table S1a. Solvent conditions for compound storage and incubations.

Compound	Stock Solvent	[Solvent] % in whole milk
Oxytetracycline (OTET)	MeOH*	0.2% MeOH
Penicillin G (PENG)	25mM K-Phos ACN ** (3:7 v:v)	0.0004% ACN(20nM) 0.004% ACN (200nM) 0.04% ACN (2,000nM
Erythromycin A (ERY)	EtOH*	100% Water***
Sulfadimethoxine (SDMX)	MeOH	100% Water***
Ketoprofen (KETO)	EtOH * 50% MeOH (unlabeled)	0.09% EtOH 0.1% MeOH
Thiabendazole (THIA)	MeOH	0.2% MeOH
Ivermectin B1a (IVR)	EtOH*	0.2% MeOH****

* As received from supplier

** Acetonitrile, ACN

*** Post purification

**** Solvent exchanged

Table S1b. Silica gel TLC conditions used for each drug extracted from milk matrix.

Drug	Abbreviation	TLC Mobile Phase	Rf
[^{3}H]Oxytetracycline	OTET (epimer)	6:35:59 H_2O:MeOH:CH_2Cl_2	0.48[a]
[^{14}C]Penicillin G	PENG	60:40 K-Phos (pH 5.5):CH_3CN	0.73
[^{14}C]Erythromycin	ERY	4:8:9 IPA:Am. Ace:EA	0.34
[^{14}C]Sulfadimethoxine	SDMX	1:1:1 chloroform:heptane:EtOH	0.54
[^{3}H]Ketoprofen	KETO	4:1 chloroform:MeOH	0.50
[^{14}C]Thiabendazole	THIA	5:3:1:1 AA:2-butanone:FA:water	0.65
[^{3}H]Ivermectin	IVR	8:1:2 toluene:EA:MeOH	0.40

[a] Related Rf's: OTET=0.29, doxycycline=0.42, tetracycline=0.38.

IPA: isopropanol
Am. Ace.: ammonium acetate
EA: ethyl acetate
EtOH: ethanol
MeOH: methanol
AA: acetic acid
FA: formic acid
CH_3CN: acetonitrile
K-Phos: 20 nM potassium phosphate buffer (pH 5.5)
CH_2Cl_2: methylene chloride

Table S2. QA/QC laboratory compositional analysis of reference standards from Eurofins DQCI of whole and skim milk samples.

	Lipid %	Total Solid %	Total N %	Total non-protein N %[b]	True Protein %	Casein Protein %
Eurofins Whole Milk[a]	3.61 ±0.50 (3.60 ± 0.40)	12.25 ± 0.54 (12.38 ± 0.58)	3.26 ± 0.26 (3.36 ± 0.29)	0.17 ± 0.02 (NV)	3.08 ± 0.26 (3.19 ± 0.28)	2.47 ± 0.23 (2.55 ± 0.22)
Min	2.57 (3.16)	11.32 (11.28)	2.84 (2.94)	0.14 (NV)	2.65 (2.75)	2.15 (2.21)
Max	4.12 (4.04)	13.07 (13.25)	3.70 (3.83)	0.21 (NV)	3.49 (3.63)	2.86 (2.98)
% Difference	0.30	-1.03	-3.24		-3.29	-2.87
Eurofins Skim Milk[a]	0.17 ± 0.06 (0.09 ± 0.03)	8.89 ± 0.15 (8.96 ± 0.10)	3.22 ± 0.06 (3.29 ± 0.07)	0.18 ± 0.01 (NV)	3.04 ± 0.06 (3.10 ± 0.06)	2.50 ± 0.05 (NV)
Min	0.05 (0.05)	8.60 (8.82)	3.16 (3.21)	0.16 (NV)	2.96 (3.03)	2.43 (NV)
Max	0.27 (0.13)	9.03 (9.05)	3.30 (3.37)	0.20 (NV)	3.12 (3.18)	2.57 (NV)
% Difference	87.53	-0.77	-1.88		-1.85	

[a] Eurofins whole and skim milk represent means of laboratory analyses of Eurofins QA/QC whole (n=12 different samples) and skim milk (n=6 different samples analyzed in duplicate) standards ± SD. For comparison, data in parentheses are Eurofins' reported values. (Samples obtained from June 2014 – January 2015)

[b] A NV means that Eurofins' values were not provided; specifically total non-protein N % in whole and skim milk, as well as casein protein % in skim milk were not given.

Table S3. [3H]OTET mean, standard deviation, and relative standard deviation values for drug data for three initial concentrations in whole milk.

OTET	Whole Milk			Skim Milk Fraction				Milk Fat Fraction				Mass Balance	Obs. Ratio	Cor. Ratio
Mean (nM)	Spike DPM	Volume (mL)	Initial nM	Total DPM	Volume (mL)	Final nM	% OTET Dose	Total DPM	Mass (g)	Final nM (nmole/kg)	% OTET Dose	Mass Balance based on Whole Milk	$[OTET]_{milk\ fat}$ / $[OTET]_{skim\ milk}$	$[OTET]_{lipid}$/ $[OTET]_{aqueous}$
0		44.05			46.53				2.23					
20	2,533,997.58	48.89	23.35	2,552,400.88	46.45	24.75	100.75	22,223.12	2.19	4.56	0.88	101.63	0.18	0.0010
200	2,527,114.63	48.79	233.33	2,552,691.56	46.46	247.48	101.01	23,045.89	2.19	47.47	0.91	101.92	0.19	0.0010
2000	2,417,965.62	48.47	2,246.98	2,397,379.81	46.11	2,341.84	99.15	22,990.47	2.22	466.44	0.95	100.10	0.20	0.0010
S.D.														
0		0.03			0.01				0.02					
20	86,790.94	0.02	0.81	62,547.90	0.02	0.60	1.01	1,035.82	0.02	0.17	0.01	1.00	0.003	0.00002
200	51,111.46	0.05	4.69	63,581.39	0.02	6.17	0.55	2,246.66	0.02	4.11	0.08	0.56	0.014	0.00002
2000	62,612.37	0.04	56.55	68,929.03	0.01	66.80	1.56	1,341.61	0.03	23.24	0.03	1.54	0.007	0.00003
%RSD														
0		0.08			0.02				0.83					
20	3.43	0.04	3.46	2.45	0.04	2.44	1.00	4.66	1.02	3.68	1.62	0.98	1.50	2.46
200	2.02	0.09	2.01	2.49	0.04	2.49	0.55	9.75	1.10	8.66	8.38	0.55	7.28	2.53
2000	2.59	0.09	2.52	2.88	0.02	2.85	1.58	5.84	1.16	4.98	3.52	1.54	3.56	2.87

Table S4. [14C]PENG mean, standard deviation, and relative standard deviation values for drug data for three initial concentrations in whole milk.

PENG	Whole Milk			Skim Milk Fraction				Milk Fat Fraction				Mass Balance	Obs. Ratio	Cor. Ratio
Mean (nM)	Spike DPM	Volume (mL)	Initial nM	Total DPM	Volume (mL)	Final nM	% PENG Dose	Total DPM	Mass (g)	Final nM (nmole/kg)	% PENG Dose	Mass Balance based on Whole Milk	$[PENG]_{milk\ fat}$ / $[PENG]_{skim\ milk}$	$[PENG]_{lipid}$/ $[PENG]_{aqueous}$
0		48.86			46.51				2.16					
20	118,498.71	48.84	19.87	118,446.16	46.56	20.84	99.97	1,344.85	2.16	5.09	1.14	101.10	0.24	0.04
200	1,196,225.01	48.90	200.34	1,194,241.77	46.61	209.84	99.84	13,867.29	2.15	52.88	1.16	101.00	0.25	0.05
2000	1,267,672.87	48.61	2,135.68	1,201,051.92	46.24	2,127.47	94.94	15,283.89	2.20	569.50	1.21	96.15	0.27	0.06
S.D.														
0		0.04			0.03				0.06					
20	1,051.66	0.02	0.17	1,154.71	0.07	0.21	1.82	57.54	0.01	0.23	0.06	1.87	0.009	0.010
200	9,525.14	0.05	1.40	672.48	0.02	0.20	0.77	324.56	0.07	0.88	0.03	0.79	0.004	0.005
2000	67,576.39	0.02	114.56	9,023.98	0.02	15.87	5.60	251.85	0.02	11.36	0.06	5.66	0.007	0.007
%RSD														
0		0.08			0.07				2.75					
20	0.89	0.03	0.86	0.97	0.16	1.02	1.82	4.28	0.30	4.58	4.97	1.85	3.57	25.6
200	0.80	0.11	0.70	0.06	0.04	0.10	0.77	2.34	3.33	1.67	2.89	0.79	1.62	9.75
2000	5.33	0.03	5.36	0.75	0.04	0.75	5.90	1.65	0.79	2.00	4.97	5.88	2.49	11.5

Table S5. [14C]ERY mean, standard deviation, and relative standard deviation values for drug data for three initial concentrations in whole milk.

ERY	Whole Milk			Skim Milk				Cream				Mass Balance	Obs. Ratio	Cor. Ratio
Mean (nM)	Spike DPM	Volume (mL)	Initial nM	Total DPM	Volume (mL)	Final nM	% ERY Dose	Total DPM	Mass (g)	Final nM (nmole/kg)	% ERY Dose	Mass Balance based on Whole	$[ERY]_{milk\ fat}$ / $[ERY]_{skim\ milk}$	$[ERY]_{lipid}$ / $[ERY]_{aqueous}$
0		48.74			46.37				2.21					
20	118,477.22	48.85	19.87	115,143.39	46.49	20.28	97.19	1,802.54	2.20	6.70	1.1	1.52	0.33	0.17
200	1,141,388.55	48.85	191.35	1,099,541.40	46.48	193.76	96.33	17,825.27	2.22	65.85	1.2	1.56	0.34	0.18
2000	1,194,781.86	48.84	2,003.56	1,145,886.77	46.43	2,021.20	95.91	18,624.82	2.23	682.56	1.2	1.56	0.34	0.18
S.D.														
0		0.07			0.07				0.04					
20	298.29	0.06	0.07	296.55	0.08	0.05	0.34	103.65	0.04	0.27	0.1	0.09	0.014	0.015
200	7,255.28	0.03	1.33	12,151.41	0.06	2.32	0.95	1,309.20	0.04	3.96	0.0	0.11	0.016	0.018
2000	7,736.38	0.04	11.81	3,893.43	0.01	6.22	0.48	703.57	0.02	21.35	0.1	0.06	0.010	0.011
%RSD														
0		0.14			0.15				1.67					
20	0.25	0.13	0.37	0.26	0.17	0.22	0.35	5.75	1.71	4.05	5.0	5.69	4.18	8.55
200	0.64	0.06	0.70	1.11	0.13	1.20	0.99	7.34	1.68	6.02	8.38	6.86	4.81	9.57
2000	0.65	0.08	0.59	0.34	0.03	0.31	0.50	3.78	0.68	3.13	3.52	3.73	2.89	5.77

Table S6. [14C]SDMX mean, standard deviation, and relative standard deviation values for drug data for three initial concentrations in whole milk.

SDMX	Whole Milk			Skim Milk Fraction				Milk Fat Fraction				Mass Balance	Obs. Ratio	Cor. Ratio
Mean (nM)	Spike DPM	Volume (mL)	Initial nM	Total DPM	Volume (mL)	Final nM	% SDMX Dose	Total DPM	Mass (g)	Final nM (nmole/kg)	% SDMX Dose	Mass Balance based on Whole Milk	$[SDMX]_{milk\ fat}$ / $[SDMX]_{skim\ milk}$	$[SDMX]_{lipid}$/ $[SDMX]_{aqueous}$
0		48.76			46.48				2.13					
20	65,496.15	48.87	21.56	60,048.63	46.47	20.79	91.69	3,774.15	2.19	27.69	5.76	97.45	1.33	1.55
200	617,810.06	48.87	203.37	568,561.70	46.51	196.66	92.03	28,210.15	2.18	208.30	4.57	96.60	1.07	1.23
2000	437,238.11	48.32	1,455.62	407,620.64	46.30	1,416.41	93.23	19,436.09	2.14	1,464.11	4.45	97.67	1.04	1.20
S.D.														
0		0.09			0.06				0.02					
20	396.34	0.04	0.14	578.07	0.08	0.22	1.40	77.63	0.07	0.38	0.12	1.47	0.018	0.021
200	12,805.91	0.03	4.31	11,885.40	0.05	4.29	0.69	740.68	0.02	3.10	0.06	0.74	0.008	0.010
2000	3,003.36	0.06	9.01	3,831.06	0.06	13.23	0.30	453.20	0.04	17.63	0.09	0.38	0.004	0.004
%RSD														
0		0.18			0.12				0.94					
20	0.61	0.07	0.65	0.96	0.17	1.04	1.53	2.06	3.01	1.39	2.16	1.51	1.38	1.38
200	2.07	0.05	2.12	2.09	0.10	2.18	0.74	2.63	1.15	1.49	1.35	0.77	0.77	0.77
2000	0.69	0.12	0.62	0.94	0.13	0.93	0.32	2.33	1.81	1.20	2.07	0.39	0.35	0.35

Table S7. [3H]KETO mean, standard deviation, and relative standard deviation values for drug data for three initial concentrations in whole milk.

KETO	Whole Milk			Skim Milk Fraction				Milk Fat Fraction				Mass Balance	Obs. Ratio	Cor. Ratio
Mean (nM)	Spike DPM	Volume (mL)	Initial nM	Total DPM	Volume (mL)	Final nM	% KETO Dose	Total DPM	Mass (g)	Final nM (nmole/kg)	% KETO Dose	Mass Balance based on Whole Milk	$[KETO]_{milk\ fat}$ / $[KETO]_{skim\ milk}$	$[KETO]_{lipid}$ / $[KETO]_{aqueous}$
0		48.78			46.28				2.25					
20	2,476,503.60	49.12	21.63	2,477,619.45	46.66	22.78	100.04	122,850.85	2.27	23.25	4.96	105.00	1.02	1.08
200	2,522,561.11	48.81	221.70	2,454,820.52	46.38	227.07	97.33	125,921.12	2.25	240.44	4.99	102.33	1.06	1.12
2000	2,484,723.50	48.50	2,197.63	2,461,259.69	46.40	2,275.68	99.05	130,593.08	2.24	2,498.95	5.26	104.31	1.10	1.16
S.D.														
0		0.03			0.17				0.06					
20	26,348.11	0.46	0.34	36,284.81	0.45	0.46	0.44	806.52	0.04	0.27	0.08	0.36	0.011	0.011
200	45,674.72	0.07	4.13	9,214.00	0.08	1.22	1.69	516.56	0.01	2.22	0.08	1.77	0.009	0.009
2000	34,120.25	0.01	30.48	35,658.63	0.04	31.15	0.08	1,743.36	0.04	24.08	0.12	0.05	0.007	0.007
%RSD														
0		0.05			0.37				2.67					
20	1.06	0.94	1.59	1.46	0.96	2.04	0.44	0.66	1.64	1.15	1.71	0.35	1.09	1.09
200	1.81	0.14	1.86	0.38	0.16	0.54	1.74	0.41	0.59	0.92	1.51	1.73	0.83	0.83
2000	1.37	0.03	1.39	1.45	0.09	1.37	0.08	1.33	1.67	0.96	2.29	0.05	0.62	0.62

Table S8. [14C]THIA mean, standard deviation, and relative standard deviation values for drug data for three initial concentrations in whole milk.

THIA	Whole Milk			Skim Milk Fraction				Milk Fat Fraction				Mass Balance	Obs. Ratio	Cor. Ratio
Mean (nM)	Spike DPM	Volume (mL)	Initial nM	Total DPM	Volume (mL)	Final nM	% THIA Dose	Total DPM	Mass (g)	Final nM (nmole/kg)	% THIA Dose	Mass Balance based on Whole Milk	$[THIA]_{milk\ fat}$ / $[THIA]_{skim\ milk}$	$[THIA]_{lipid}$ / $[THIA]_{aqueous}$
0		48.78			46.30				2.27					
75	167,052.89	48.88	77.37	130,829.67	46.41	63.80	78.32	37,210.93	2.28	370.14	22.28	100.59	5.80	6.65
200	446,844.67	48.74	207.49	342,970.75	46.23	167.90	76.75	98,407.04	2.29	972.46	22.02	98.78	5.77	6.64
2000	441,839.65	48.57	2,059.27	343,840.96	46.03	1,691.03	77.82	97,482.27	2.28	9,688.62	22.06	99.88	5.67	6.56
S.D.														
0		0.01			0.04				0.03					
75	3,131.31	0.03	1.40	2,463.09	0.02	1.18	0.26	232.87	0.02	1.15	0.29	0.36	0.125	0.146
200	11,860.85	0.20	4.66	9,553.66	0.20	4.00	0.78	2,450.75	0.02	19.77	0.12	0.90	0.020	0.024
2000	3,085.39	0.01	13.87	3,473.50	0.06	15.00	0.64	904.36	0.01	47.11	0.26	0.49	0.078	0.092
%RSD														
0		0.03			0.08				1.33					
75	1.87	0.07	1.82	1.88	0.05	1.85	0.33	0.63	0.87	0.31	1.31	0.36	2.15	2.20
200	2.65	0.41	2.25	2.79	0.43	2.38	1.02	2.49	0.67	2.03	0.55	0.91	0.35	0.36
2000	0.70	0.03	0.67	1.01	0.13	0.89	0.82	0.93	0.49	0.49	1.17	0.49	1.37	1.40

Table S9. [3H]IVR mean, standard deviation, and relative standard deviation values for drug data for three initial concentrations in whole milk.

IVR	Whole Milk			Skim Milk Fraction				Milk Fat Fraction				Mass Balance	Obs. Ratio	Cor. Ratio
Mean (nM)	Spike DPM	Volume (mL)	Initial nM	Total DPM	Volume (mL)	Final nM	% IVR Dose	Total DPM	Mass (g)	Final nM (nmole/kg)	% IVR Dose	Mass Balance based on Whole Milk	$[IVR]_{milk\ fat}$ / $[IVR]_{skim\ milk}$	$[IVR]_{lipid}$ / $[IVR]_{aqueous}$
0		48.74			46.39				2.10					
20	1,215,646.67	48.74	22.47	151,110.96	46.07	2.95	12.43	972,467.32	2.13	411.69	79.99	92.42	139.61	241.91
200	1,147,099.45	48.75	211.97	145,576.85	46.16	28.42	12.69	928,781.10	2.15	3,901.69	80.97	93.66	137.54	236.21
2000	1,148,546.53	48.48	2,133.98	156,822.50	46.49	303.93	13.67	935,701.69	2.13	39,575.20	81.42	95.09	130.21	217.05
S.D.														
0		0.03			0.13				0.02					
20	6,853.38	0.03	0.13	9,505.41	0.63	0.16	0.72	32,627.74	0.04	14.21	2.54	3.10	8.281	22.81
200	12,518.37	0.08	2.10	6,696.57	0.49	1.37	0.49	34,214.01	0.09	57.44	3.06	2.63	7.716	20.90
2000	42,078.55	0.57	55.26	1,273.45	0.02	2.43	0.50	54,937.56	0.01	2,370.69	1.83	1.34	7.611	19.04
%RSD														
0		0.06			0.27				1.09					
20	0.56	0.06	0.58	6.29	1.36	5.45	5.79	3.36	1.93	3.45	3.18	3.35	5.93	9.43
200	1.09	0.17	0.99	4.60	1.07	4.84	3.85	3.68	4.38	1.47	3.78	2.81	5.61	8.85
2000	3.66	1.18	2.59	0.81	0.04	0.80	3.65	5.87	0.54	5.99	2.25	1.41	5.85	8.77

Table S10. Linear regression analysis of log $[Drug]_{milk\ fat}/[Drug]_{skim\ milk}$ for different dose levels (20, 200, 2000 nM).

Drug	Linear Regression[a]
OTET	NS
PENG	p=0.004, 0.4% change from 20 to 2000 nM
ERY	NS
SDMX	NS
KETO	p=0.002, 0.3% change from 20 to 2000 nM
THIA	NS
IVR	NS

[a] NS is not significant.

Table S11. Values used for regression analyses of log [Drug]milk fat/[Drug]skim milk vs log P and log D (pH 6.8) for all data used in graphs and for calculations from multiple databases.

Drug	log P Chemspider[1]	pKa Drug Bank[1]	log D (pH 6.8) Drug Bank[1] pKa	log D pH 6.8 (lowest relevant pKa value)[2]	log D pH 6.8 (highest relevant pKa value)[2]
OTET	-1.5	7.75	-2.5	-2.12 (7.3)	-2.50 (7.75)
PENG	1.67	3.53	-1.6	-2.68 (2.45)	-1.60 (3.53)
ERY	2.83	8.38	1.24	1.47 (8.14)	0.53 (9.1)
SDMX	1.48	6.91	1.23	0.53 (5.9)	1.23 (6.91)
KETO	2.81	3.88	-0.11	-0.11(3.88)	0.46 (4.45)
THIA	2.93	4.08	2.93	2.93 (3.4)	2.93 (4.7)
IVR	6.61	12.47	6.61	6.61 (12.42)	6.61 (12.47)

[1]EMBL pKas accessed on 2-9-2015 and Drug Bank pKas accessed on 2-11-2015. Log P accessed from Chemspider on 1-28-2015. Log D at pH 6.8 calculated using $\log D_{acid} = \log P + \log[1/(1+10^{pH-pKa})]$ or $\log D_{base} = \log P + \log[1/(1+10^{pKa-pH})]$.

[2] Values in parentheses are the pKa value used to calculate log D.

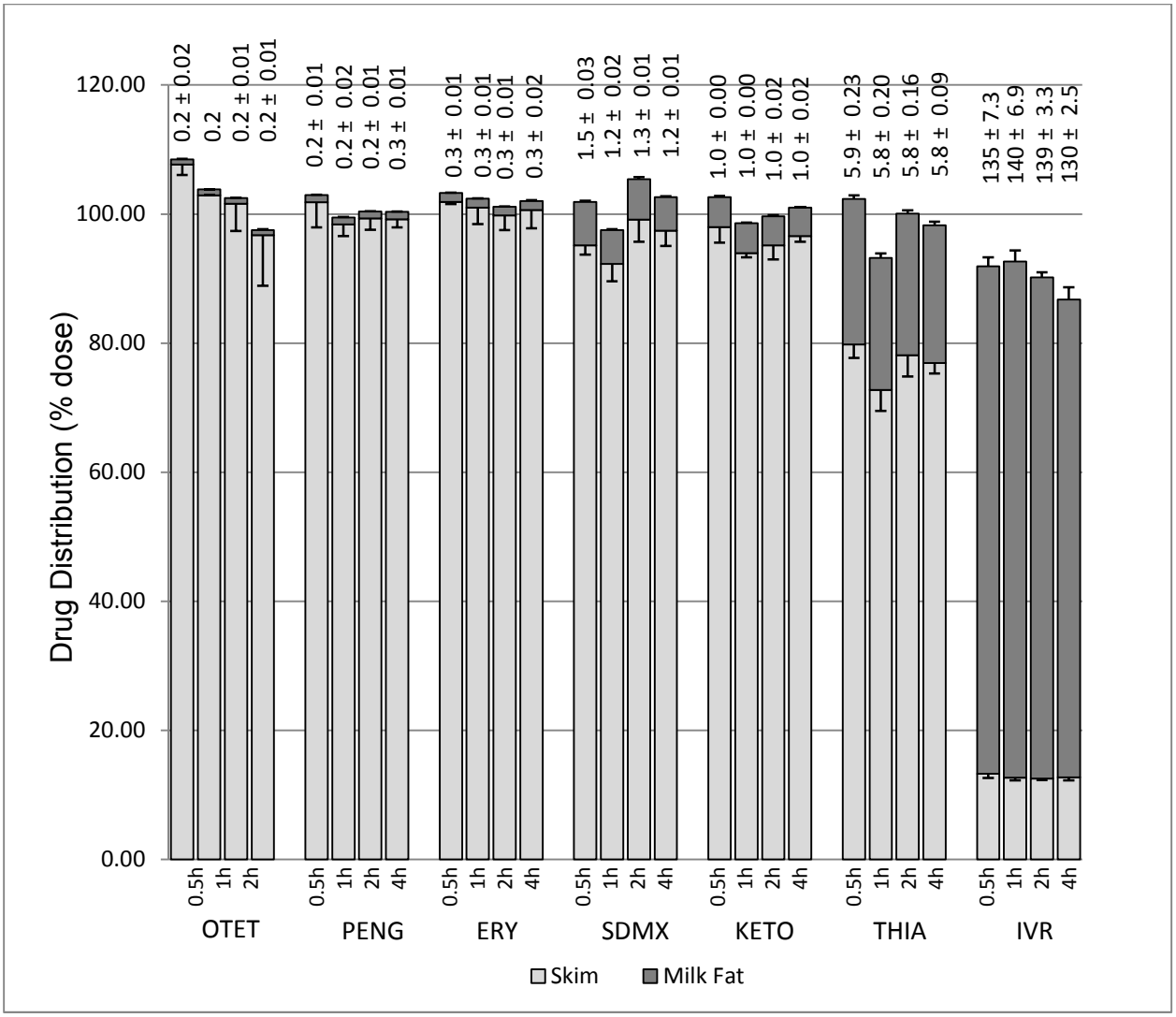

Figure S1. Drug distribution into skim milk and milk fat fractions from fortified whole milk at four different equilibration times. Bars represent mean values (n=3) of each fraction ± SD, with sum of the two fractions representing recovery. No error bar for OTET 1hr, as n=2. Values above bars represent concentration ratios of $[Drug]_{milk\ fat}/[Drug]_{skim\ milk}$.